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APPLICATION FOR LETTERS PATENT

for

YERSINIA SPECIES COMPOSITIONS

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TITLE OF THE INVENTION

YERSINIA SPECIES COMPOSITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] Pursuant to 35 U.S.C. § 119(e), this application claims the benefit of U.S. Provisional Application 60/444,076, filed January 31, 2003.

TECHNICAL FIELD

[0002] The invention relates generally to the field of biotechnology, and more particularly to compositions for eliciting an immune response including an isolated or recombinant YscF or an epitope thereof that provides protection against infections caused by members of the genus *Yersinia*.

BACKGROUND

[0003] *Yersinia pestis* causes a rapidly progressing disease in humans with a high mortality rate. Due to the severe nature of the disease and its ability for aerosol transmission, a better vaccine for the disease caused by *Y. pestis*, the plague, is desirable. Current efforts for vaccine development have focused on two proteins: LcrV and the F1 antigen (25). The best results to date have been obtained by using a combination of recombinant LcrV and F1 subunits (25). This vaccine demonstrates protection against both pneumonic and systemic forms of plague (25). One of the potential limitations of this vaccine is that the F1 antigen is not required for full virulence of *Y. pestis* as F1-negative strains have the same LD_{50} value as F1-positive strains (6-8, 27). While the recombinant sub-unit vaccine is effective and offers protection against F1 minus strains of *Y. pestis*, the inclusion of other antigens with the LcrV-F1 vaccine could improve the ability of the resulting vaccine to offer protection against multiple *Y. pestis* strains, or new antigens could be developed as separate vaccine candidates. Another *Yersinia* protein that has been shown to provide some protection is YopD (25).

[0004] The type III secretion apparatus is encoded on the low-calcium response (LCR) virulence plasmid, pCD1 in strain KIM (20) of *Y. pestis*. The type III secretion apparatus is a conserved virulence mechanism that is absolutely required for virulence of *Y. pestis* (19). YscF (*See*, SEQ ID NOS: 1 and 2 for the amino acid sequence and the *yscF* sequence, respectively) is a

surface localized protein that is required both to secrete Yops and to translocate toxins into eukaryotic cells (1, 10, 12). The type III secretion apparatus and YscF are also encoded for by the virulence plasmids of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogenic bacteria transmitted by the oral route and cause a range of gastrointestinal diseases collectively referred to as yersiniosis. The nucleic acid sequence for YscF of *Y. pseudotuberculosis* and the amino acid sequence for YscF of *Y. pseudotuberculosis* are substantially similar to the *yscF* gene and YscF protein of *Y. pestis* based on homologies and comparisons of other proteins of the type III secretion complex. The nucleic acid sequence encoding YscF of *Y. enterocolitica* includes SEQ ID NO: 3 and the amino acid sequence contains SEQ ID NO: 4. An alignment of the YscF proteins from these organisms is illustrated in FIG. 1.

[0005] One report speculates that YscF polymerization is required for a YscF needle to puncture eukaryotic cell membranes (12). Other researchers suggest that YscF and its homologs function to provide a base that a translocon complex is built upon, or that YscF builds a conduit from the bacterium to the eukaryotic membrane (4). This suggestion seems more likely given that other proteins such as YopB, YopD, and LcrV are also required for translocation into eukaryotic cells (9, 11, 13, 17, 18, 21, 23, 24). However, the exact function of YscF remains unknown.

[0006] Other pathogenesis-related type III secretion systems possess homologs to YscF. In pathogenic *Salmonella* and *Shigella*, the YscF homologs (PrgI (*See*, SEQ ID NOS: 5 and 6 for the amino acid sequence and nucleic acid sequences, respectively) and MxiH, respectively, (*See*, SEQ ID NOS: 7 and 8 for the amino acid sequence and nucleic acid sequence, respectively)) have been demonstrated to form a needle structure that protrudes from the surface of bacterial cells (2, 15, 16). The best characterized homolog of YscF is EscF (*See*, SEQ ID NOS: 9 and 10 for the amino acid sequence and nucleic acid sequence, respectively) of enteropathogenic *E. coli* (EPEC). EscF is required for "attaching and effacing" (A/E) lesion formation on the intestinal mucosa and for type III secretion of effector proteins (5, 22, 29). EscF is thought to be a structural component of the needle complex on the bacterial surface as it binds EspA, the major component of a filamentous surface organelle, and is required for formation of the EspA filaments (5, 22, 29). However, this surface localization has never been directly visualized and the only EscF antiserum generated was unable to recognize the native protein (29).

[0007] Based on the fact that YscF is thought to be a surface-expressed protein in the pathogens of *Yersinia* and is required for virulence, it was determined whether YscF could serve as a protective antigen against experimental infection with pathogens of *Yersinia*.

SUMMARY OF THE INVENTION

[0008] In one exemplary embodiment, a composition of matter comprising YscF of a *Yersinia* origin is disclosed. The composition of matter may comprise isolated or recombinant YscF; a recombinant vector including the nucleic acid associated with isolated or recombinant YscF; synthetic YscF; a nucleic acid encoding the isolated or recombinant YscF; a recombinant nucleic acid which comprises a nucleotide sequence originating from the genome of *Yersinia*; a polypeptide having an amino acid sequence originating from a protein of *Yersinia*, the polypeptide being produced by a cell capable of producing it due to genetic engineering with appropriate recombinant DNA; an isolated or synthetic antibody which specifically recognizes a part or epitope of the isolated or recombinant YscF; or a recombinant vector which contains nucleic acid comprising a nucleotide sequence coding for a protein or antigenic peptide associated with isolated or recombinant YscF.

[0009] In another exemplary embodiment, a recombinant nucleic acid, more specifically recombinant DNA, which comprises a nucleotide sequence encoding for isolated or recombinant YscF, as shown in SEQ ID NO: 11 is disclosed (*see*, SEQ ID NO: 12 for the amino acid sequence encoded by SEQ ID NO: 11). In a further embodiment, a pharmaceutical composition including the nucleotide sequence encoding for isolated or recombinant YscF is disclosed. Use of the recombinant nucleic acid encoding the isolated or recombinant YscF for the prophylaxis of an animal is also disclosed.

[0010] In an additional embodiment, a peptide comprising an isolated or recombinant YscF amino acid sequence is disclosed. The YscF proteins disclosed herein are capable of conferring protection to an animal against a pathogen or *Yersinia* origin. In a further embodiment, a pharmaceutical composition including the isolated of recombinant YscF is disclosed. Use of the isolated or recombinant YscF for the prophylaxis of an animal is also disclosed.

[0011] In yet another embodiment, a vaccine for vaccinating animals, in particular mammals, to protect them against infections caused by pathogens of *Yersinia* origin, such as *Y.*

pestis, *Y. pseudotuberculosis* and *Y. enterocolitica* is disclosed. The vaccine comprises isolated or recombinant YscF; a recombinant vector which contains the nucleic acid coding for a protein or antigenic peptide associated with isolated or recombinant YscF; an antigenic part or epitope of isolated or recombinant YscF; or a peptide mimicking an antigenic component of isolated or recombinant YscF; together with a suitable carrier or adjuvant.

[0012] Use of a composition comprising an isolated or recombinant YscF for the manufacture of a medicament for the treatment of a mammal infected with a *Yersinia* pathogen, such as *Y. pestis*, *Y. pseudotuberculosis* or *Y. enterocolitica* is further disclosed. In a further aspect, the invention discloses the use of a composition comprising antibodies or fragments thereof that bind to isolated or recombinant YscF for the manufacture of a medicament for the treatment of a mammal infected with a *Yersinia* pathogen, such as *Y. pestis, Y. pseudotuberculosis* or *Y. enterocolitica* or prevention of such an infection.

[0013] In a further exemplary embodiment, a diagnostic kit for detecting antibodies generated against isolated or recombinant YscF in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue (*i.e.*, lymph nodes), originating from an animal, in particular a mammal is disclosed. The diagnostic kit comprises an antibody or fragment thereof that binds to the isolated or recombinant YscF or a fragment thereof, and suitable detection means of an antibody detection assay.

[0014] The invention also discloses a diagnostic kit for detecting an antigen or epitope originating from YscF in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, comprising an antibody or fragment thereof that recognizes a part or epitope of YscF, and suitable detection means of an antigen detection assay.

[0015] In a further embodiment, a process for diagnosing whether an animal, in particular a mammal, is carrying the antibodies directed against isolated or recombinant YscF is disclosed. The process comprises preparing a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from the animal, and examining whether the biological sample contains the isolated or recombinant YscF antigen, or an antibody specifically recognizing the isolated or recombinant YscF, the YscF being associated with infections caused by bacteria of the *Yersinia* species.

[0016] A method for vaccinating a mammal comprising cloning a nucleic acid sequence encoding an isolated or recombinant YscF of a *Yersinia* origin or a homolog thereof into an expression vector is disclosed in another embodiment. The method further includes inducing the expression of the nucleic acid and collecting the isolated or recombinant YscF or homologs thereof. The isolated or recombinant YscF or homolog thereof is administered to an animal, such as a mammal, to generate an immune response against the isolated or recombinant YscF or homolog thereof.

[0017] A process for manufacturing a composition for use in vaccinating animals is also disclosed. The process comprises cloning a nucleic acid sequence encoding an isolated or recombinant YscF or a homolog thereof into an expression vector. The process further includes inducing the expression of the nucleic acid and collecting the isolated or recombinant YscF. The isolated or recombinant YscF or homolog thereof is mixed with a pharmaceutically acceptable excipient to produce the composition.

[0018] In an additional embodiment, a method for generating an immune response is disclosed. The method includes cloning a nucleic acid sequence encoding an isolated or recombinant YscF or a homolog thereof into an expression vector. The method further includes inducing the expression of the nucleic acid and collecting the isolated or recombinant YscF or homolog thereof. The isolated or recombinant YscF or homolog thereof is administered to a subject to generate the immune response in the subject.

[0019] A method of collecting antibodies generated against an epitope of an isolated or recombinant YscF or homolog thereof is disclosed in a further embodiment. The method includes cloning a nucleic acid sequence encoding the isolated or recombinant YscF or a homolog thereof into an expression vector. The method further includes inducing the expression of the nucleic acid and collecting the isolated or recombinant YscF or homolog thereof. The isolated or recombinant YscF or homolog thereof is administered to a subject. The isolated or recombinant YscF or a homolog thereof is immobilized on a substrate and serum collected from the subject is added to the substrate such that antibodies in the serum directed against the isolated or recombinant YscF or a homolog thereof adhere to the immobilized protein.

[0020] In an additional embodiment, a peptide corresponding to an epitope of the isolated or recombinant YscF or homolog thereof to which an antibody binds is disclosed. A composition or

vaccine including the epitope of the isolated or recombinant YscF or homolog thereof to which an antibody binds is further disclosed. The use of a composition comprising the epitope of the isolated or recombinant YscF or homolog thereof to which an antibody binds for the manufacture of a medicament for the treatment of a mammal infected with a pathogen of *Yersinia* origin, such as such as *Y. pestis*, *Y. pseudotuberculosis* or *Y. enterocolitica* is further disclosed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 is a sequence alignment of peptide sequences of the YscF protein from various *Yersinia* bacteria including SEQ ID NOS: 4, 13, 17, 18 and 19.

[0022] FIG. 2 is a map of the pJM119 plasmid (SEQ ID NO: 13).

[0023] FIG. 3 is a map of the pET24b plasmid (SEQ ID NO: 14).

[0024] FIGS. 4A and 4B illustrate recovered His-tagged YscF protein run on a SDS-PAGE.

[0025] FIG. 5 represents the presence or absence of YscF protein in the culture supernatants of various *Y. pestis* strains.

BEST MODE OF THE INVENTION

[0026] The embodiments described herein disclose the successful immunization of mice with isolated or recombinant YscF. Previous attempts to immunize mice against subsequent challenge with *Y. pestis* have failed. For instance, Hill et al. immunized mice with YscF, but were not able to show protection in the mice against subsequent challenge with *Y. pestis*. (*See*, Hill et al., *Immunological characterization of sub-units of the Yersinia type III secretion apparatus*, Abstract at the 8th International Symposium on Yersinia. Sept. 4-8, 2002. Turku, Finland.). Thus, the present invention discloses a surprising discovery that isolated or recombinant YscF is able to protect subjects against subsequent challenge with a pathogen of *Yersinia* origin. The immunization disclosed herein results in a high anti-YscF titer and protection against challenge with a pathogen of *Yersinia* origin. The embodiments described herein disclose that YscF provides protection against challenge with a pathogen of *Yersinia* origin and, thus, is a vaccine candidate. The isolated or recombinant YscF may also be used in conjunction with the other known plague antigens.

[0027] The phrase "suitable excipient" as used herein means that an active ingredient can be formulated, for example, with the conventional generally non-toxic, well-known pharmaceutically acceptable carriers (*e.g.*, sterile water, saline solution and other acceptable carriers) for making suitable pharmaceutical compositions. The suitable excipient may also include adjuvants as described herein. A person of ordinary skill in the art will recognize that a suitable excipient, examples of which are provided herein, is an art recognized term.

[0028] The vaccine may also comprise compounds including an adjuvant activity. Adjuvants are non-specific stimulators of the immune system and enhance the immune response of the animal host to the vaccine. Examples of adjuvants that may be used include, but are not limited to, incomplete Freund's adjuvant, Freunds. Complete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, ISCOMs (immune stimulating complexes), Saponins, mineral oil, vegetable oil, Carbopol, Alhydrogel, and Ribi. Adjuvants suitable for mucosal application include *E. coli* heat-labile toxin or *Cholera* toxin. Other suitable adjuvants include aluminum hydroxide, aluminum phosphate or aluminum oxide, oil-emulsions or vitamin-E solubilisate. The vaccine may also include preservatives to increase the shelf live of the vaccine.

[0029] In the exemplary embodiments herein, the vaccines or compositions including the isolated or recombinant YscF of *Yersinia* origin or homologs thereof may also include pharmaceutically acceptable carriers including, but not limited to, water, culture fluid in which the bacteria were cultured, a solution of physiological salt concentration, stabilizers such as SPGA, carbohydrates (*e.g.*, sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk, and buffers (*e.g.*, phosphate buffer). When stabilizers are added to the vaccine, the vaccine is suitable for freeze-drying. Accordingly, in another exemplary embodiment, the vaccine may be in a freeze-dried form as is known by those of ordinary skill in the art.

[0030] The vaccines of the exemplary embodiments may be administered to humans or animals *inter alia* intraperitoneally, intranasally, intradermally, subcutaneously, orally, by aerosol or intramuscularly. As known in the art, the vaccine may be in a unit dosage form and provided in sterile form in a sealed container. The dosage administered to the animal will vary depending on the age, weight and animal vaccinated, as well as the mode of administration and the frequency of administrations employed. Regimens for inducing an immune response including dose and therapy

may be guided by the initial response of the animal to the first vaccine dose and clinical judgment as known by those of ordinary skill in the art.

[0031] Types of animals that the vaccine may be administered to include any mammal, such as humans, pigs, mice, prairie dogs, cats, dogs and rats or other animals. The vaccine may be used to generate a "herd immunity" in a population or a sub-population of animals. As known in the art, the phrase "herd immunity" refers to the effect achieved when enough individuals of the population or sub-population are vaccinated such that the particular disease is not able to spread through the population or sub-population. Thus, the immunized individuals are protected as are the non-immunized individuals since the disease cannot effectively spread through the population or sub-population. Accordingly, the vaccine has utility in a public health program designed to help prevent the transmission of infections caused by pathogens of *Yersinia* origin.

[0032] The phrase "pathogens of *Yersinia* origin" will be used to refer to members of the genus *Yersinia* that cause disease including, but not limited to, *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* which encode substantially identical and functionally equivalent YscF proteins. As described herein, the term "YscF" will be used to refer to the YscF protein originating from any of *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica* unless otherwise specified. The YscF proteins of *Y. pestis and Y. enterocolitica* include substantially similar sequences as indicated in the alignment of FIG. 1. Further, since many proteins of the type III secretion complex of both *Y. pestis* and *Y. pseudotuberculosis* are substantially similar, YscF from *Y. pestis* and *Y. pseudotuberculosis* are considered to be substantially similar.

[0033] The term "protective" or "conferring protection" as used herein with reference to a protein will be used to refer to the ability of the protein to increase the lethal dose of pathogenic bacteria required to kill 50% of hosts infected with the pathogenic bacteria after administration of the protein to the host.

[0034] As used herein, the term "recombinant YscF" will be used to refer to a YscF protein that includes amino acid residues in addition to or different than wild-type YscF. For instance, His-tagged YscF is a recombinant YscF (*see,* SEQ ID NOS: 11 and 12 for the nucleic acid and amino acid sequences of His-tagged YscF, respectively).

[0035] In addition to the peptides, vaccines and compositions including isolated or recombinant YscF or homologs thereof described herein, peptides functionally and

immunologically related to the isolated or recombinant YscF or homologs thereof that possess the same functions and immunologic properties as the isolated or recombinant YscF or analogs thereof are further disclosed. For instance, amino acid substitutions in the peptide may not substantially alter the biological and immunological activities of the protein and have been described, *e.g.*, Neurath et al. in "The Proteins" Academic Press, New York (1979). Amino acid replacements which occur frequently in evolution and do not alter the function or immunological activity of the protein include *inter alia*, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (*see*, Dayhof. M.C., Atlas of protein sequence and structure, Nat. Biomed, Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Other amino acid substitutions that often do not alter the function of immunogenicity of proteins include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn/Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Based on this information, Lipman and Pearson have developed a method for rapid and sensitive protein comparison (*Science*, 227, 1435-1441, 1985) and determining the functional similarity between homologous proteins. Accordingly, amino acid substitutions which do not alter the function or immunological properties of the isolated or recombinant YscF or homologs thereof, are encompassed by the present invention.

[0036] In addition to the nucleotide sequences, vaccines or compositions including the nucleotide sequences encoding for the isolated or recombinant YscF or homologs thereof, nucleotide sequences having substantially similar functions as the nucleotides encoding the isolated or recombinant YscF or homologs thereof are further disclosed. For instance, as known in the art, the degeneracy of the genetic code and the "wobble" hypothesis allows for nucleotide substitutions to occur and, yet, the altered nucleotide sequence encodes a protein having a substantially similar function or immunogenicity as the proteins encoded by original nucleotide sequence since some amino acids are encoded by more than one codon.

[0037] Further, as previously described herein, some amino acid substitutions may not alter the function or immunological properties of the protein. For instance, single nucleotide polymorphisms, allelic variants, insertions and deletions may have different nucleotide sequences from those disclosed herein, but still encode isolated or recombinant YscF proteins or homologs thereof. Accordingly, nucleotide substitutions in the nucleic acids of the present invention which do not substantially alter the peptide sequence of the isolated or recombinant YscF proteins or homologs thereof and nucleotide substitutions which encode for proteins having substantially the

same function or immunological properties as the isolated or recombinant YscF proteins or homologs thereof are encompassed by the present invention. Thus, nucleic acid sequences that hybridize to the nucleic acid sequences encoding the isolated or recombinant YscF or homologs thereof under highly stringent conditions, such as high salt conditions, are within the scope of the present invention.

Example I.

Expression and purification of HT-YscF.

[0038] Expression and purification of HT-YscF. Plasmid pJM119 (*See*, FIG. 2 and SEQ ID NO: 13) was constructed by cloning a *Bam*HI- and *Xho*I-cleaved PCR product into pET24b (SEQ ID NO: 14), a cloning vector commercially available from Novagen of Madison, Wisconsin. (*See*, FIG. 3). The primers used to amplify *yscF* were HT-YscF Start (5' CGG GAT CCG ATG AGT AAC TTC TCT GGA TTT 3') (SEQ ID NO: 15) and HT-YscF Stop (5' CCG CTC GAG TGG GAA CTT CTG TAG GAT GCC 3') (SEQ ID NO: 16). *E. coli* BL21(DE3) (commercially available from Novagen of Madison, Wisconsin) harboring pJM119 was grown in one liter of LB broth containing carbenicillin at 37°C. Expression of HT-YscF was induced after 2h of growth with 0.3 mM IPTG and incubated until the A_{550} reached ~1.0.

[0039] Cells were harvested by centrifugation and disintegrated by passage through a French pressure cell at 20,000 lb/in^2. Subsequent to disintegration, the extracts were clarified by centrifugation at 3200 x *g* for 20 min at 4°C. Affinity purification of His-tagged YscF (HT-YscF) was performed using Talon resin (Clontech of Palo Alto, California) as described by the manufacturer. Purity of the recovered protein was estimated by SDS-PAGE on a 15% (wt/vol) gel followed by staining with Gelcode Blue (Pierce of Rockford, Illinois). The purified protein ran as multiple bands on the gel. A band that corresponded to the predicted size of HT-YscF was the dominant species and other larger bands could also be visualized (*See*, FIGS. 4A and 4B). Based on the sizes of the larger bands and the fact that they are recognized by the penta-His antibodies, it is likely that the larger bands represent dimers and other multimers of YscF. This is not surprising as YscF and its homologs are known to form multimeric structures.

Example II.

[0040] The His-tag is removed from the isolated His-tagged YscF protein using known processes. For instance, treatment of the His-tagged YscF protein with Staphlylococcal peptidase I, (Protease V8) which is commercially available from Worthington Biochem of Lakewood, New Jersey is performed. (*See*, Birktoft J.J., *et al.*, *Glutamyl endopeptidases*, Methods of Enzymology (1994) 244: 114126). Cleavage of the His-tagged YscF with Staphlylococcal peptidase I results in breaking of the peptide bond between amino acid 89 and 90 of the His-tagged YscF (SEQ ID NO: 12), and results in the YscF (SEQ ID NO: 1) with an additional two amino acids, leucine and glutamate, on the carboxyl terminus. Staphlylococcal peptidase I is also referred to as glutamyl endopeptidase.

Example III.

[0041] In other exemplary embodiments, the nucleic acid sequences encoding YscF from *Y. enterocolitica* (SEQ ID NO: 3) or *Y. pseudotuberculosis* or the homologs of YscF, *i.e.*, the nucleic acids encoding PrgI, MxiH and EscF (SEQ ID NOS: 6, 8 and 10), respectively, are cloned and expressed. In this manner, His-tagged YscF from *Y. enterocolitica* or *Y. pseudotuberculosis* or His-tagged PrgI, MxiH and EscF proteins are collected and mixed with a suitable excipient to form a pharmaceutical composition. The pharmaceutical composition is used to immunize mice.

Example IV.

Immunization protocol.

[0042] Active immunization of outbred mice followed by challenge with *Y. pestis* KIM5. KIM5 is a strain of *Y. pestis* that when administered to a mammal, causes an infection in substantially the same manner as wild-type *Y. pestis*. For challenge with *Y. pestis*, 6- to 8-week-old female Swiss-Webster mice were immunized i.p. (intraperitoneally) with 40 μg/mouse His-tagged YscF or phosphate-buffered saline (3) PBS (control mice) emulsified 1:1 with complete Freund's adjuvant (CFA). Experimental mice were boosted with 40 μg/mouse His-tagged YscF in incomplete Freund's adjuvant (IFA) at two weeks and 20 μg/mouse His-tagged YscF in IFA at 4 weeks post-immunization. Negative control mice received PBS emulsified with IFA. Two weeks after the final immunization, groups of 10 mice were challenged i.v. (intravenously) via the retro-

orbital sinus with 10^1 to 10^6 CFU (colony forming units) *Y. pestis* KIM5 in PBS. The mice were observed for 19 days after challenge, and the average doses required to kill 50% of the mice (LD_{50}) for the treatment groups were calculated using the extrapolation method of Reed and Muench (26).

Example V.

[0043] The vaccine including the isolated or recombinant YscF is combined with other antigens protective against infections with bacteria, such as *Yersinia* bacteria, including LcrV, F1 antigen, YopD and a live attenuated *Yersinia* bacterium (EV76 strain), a live recombinant carrier bacterium including a nucleic acid encoding the isolated or recombinant YscF, an inactive or killed whole cell *Yersinia* bacterium and any combinations thereof. In a further embodiment, the vaccine including the isolated or recombinant YscF is combined with homologs of YscF including PrgI, MxiH, EscF and mixtures thereof.

Example VI.

[0044] In another exemplary embodiment, the nucleic acids encoding the isolated or recombinant YscF of *Yersinia* origin or homologs thereof are introduced into an animal through a microorganism (*e.g.* a bacterium or a virus) in such a way that the recombinant microorganism is able to replicate and, thus, express the polypeptide encoded by the nucleic acids and elicit an immune response in the infected animal. (*See*, M.A. Berry et al. in *Nature* (1995), 377; pp/ 632-635 discloses the preparation of vaccines using nucleic acid molecules).

[0045] The vaccines including the nucleic acid encoding the isolated or recombinant YscF or homologs thereof are manufactured by transforming an expression vector including the nucleic acid encoding the isolated or recombinant YscF or homologs thereof into a cell, multiplying the expression vectors and injecting purified expression vectors into a subject. As known by those of ordinary skill in the art, nucleic acid vaccines may comprise expressible DNA or mRNA which may be delivered to cells of the animal to be vaccinated. When the nucleic acid encoding the isolated or recombinant YscF or homologs thereof is operably linked to a promoter expressible in the animal to be vaccinated, the cells of the animal will express the nucleic acid and, thus, include the capability to induce a cell mediated immune response, a humoral immune response or a combination thereof.

Example VII.

[0046] Mice that were immunized with YscF demonstrated a 134-fold increase in the calculated LD_{50} value as compared to PBS immunized mice (Table 1). The increased LD_{50} value demonstrates that immunization with YscF protects mice from lethal challenge with *Y. pestis* KIM5 (Table 1). This result demonstrates that YscF can be developed as a novel vaccine for pathogens of *Yersinia* origin, such as *Y. pestis,* or could serve as another antigen in a multivalent *Yersinia* vaccine including YscF, the F1 antigen, LcrV, and combinations thereof. Based on the high degree of homology among YscF proteins originating from strains of *Y. pestis* and *Y. enterocolitica* as illustrated in FIG. 1, the protection conferred by YscF against *Y. pestis* is also expected to confer protection against infections with *Y. enterocolitica* which includes a substantially similar type III secretion system. Further, since many proteins of the type III secretion complex of both *Y. pestis* and *Y. pseudotuberculosis* are substantially similar, the protection conferred by YscF against *Y. pestis* is also expected to confer protection against infections with *Y. pseudotuberculosis.*

Table 1

Immunogen	anti-YscF GMT*	LD_{50}	Fold increase in survival
PBS	< 1:400	159	–
HT-YscF	1:40,000	21,344	134

*Geometric mean titer

Example VIII.

Characterization of the antibody response to HT-YscF.

[0047] Characterization of the antibody response to HT-YscF. Flat-bottom, 96-well Nunc Maxisorp immunoplates (Fisher Scientific, Pittsburgh, PA) were coated with 100 µl of HT-YscF solution (4 µg/ml in Binding solution (0.1 M NaH_2PO_4, ph 9.0) at room temperature for 2 h (or overnight at 4°C). The wells were blocked with 200 µl/well blocking buffer (1% bovine serum albumin in TTBS (tris-buffered saline (3) + 0.5% Tween 20) and washed with TTBS. Test sera

were serially diluted in blocking buffer and 100 µl of each dilution was added to duplicate wells that were incubated for 2 h at RT (or overnight at 4°C). The plates were washed and incubated for 2 h at RT with alkaline-phosphatase-conjugated anti-mouse secondary antibody. The high antibody response observed against HT-YscF is evidence that YscF is not only expressed during the course of an infection with pathogens of *Yersinia* origin, but also that YscF is in a location accessible to antibodies, such as on the bacterial surface.

[0048] For quantitation of YscF-specific immunoglobulin isotypes and subclasses, the plates were coated with alkaline-phosphatase-labeled anti-mouse isotype-specific antibody (1:400 in blocking buffer; Southern Biotech, Birmingham, AL). The wells were washed and 75 µl 3mM para-nitro phenyl phosphate (p-NPP) was added to each well. The plates were incubated for 15 min at RT (room temperature) and the reaction was stopped by the addition of 50 µl of 1.5 M NaOH to each well. A_{405} was measured with a Thermo Max kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA) to monitor the cleavage of p-NPP. Antibody titers were determined as reciprocal numbers of the highest serum dilution that displayed values for optical density twofold higher than the value of the control serum.

[0049] Anti-YscF antibody titers were determined two weeks following the last immunization, prior to challenge. The YscF-specific antibody titers of PBS-immunized mice were below the ELISA assay baseline of 400 (Table 1), as was the pre-immune serum (data not shown). However, the HT-YscF immunized mice reached a GMT (geometric mean titer) of 40,000 (Table 1). The IgG titer was very high, especially the IgG1 and IgG2b subclasses and the antibody response consisted primarily of antibodies possessing kappa light chains. Interestingly, Titball et al. showed that IgG1 titers to the F1-LcrV chimera correlated very well with protection against pneumonic plague (28). This suggests that YscF may afford protection against pneumonic challenge as well as against systemic challenge.

Example IX.

[0050] Derivatives of *Yersinia pestis* KIM8-3002 (KIM5 pPCP1-minus, Sm^r) were grown in a chemically defined medium (17) at 26°C for 2 h in the presence (lanes 1, 3, and 5) (FIG. 5) or absence of calcium (lanes 2, 4, and 6) (FIG. 5) or the presence of arabinose (lanes 3 and 4) (FIG. 5). pPCP1 is a plasmid originating from *Y. pestis* and has the Medline Accession No. AL109969. (*See*,

Parkhill et al., Genome sequence of *Yersinia pestis*, the causative agent of the plague, Nature 413 (6855), 523-527 (2001)). Lanes 1 and 2 contain *Y. pestis* KIM8-3002. Lanes 3 and 4 contain *Y. pestis* KIM8-3002 expressing YscF from pBAD18-YscF (SEQ ID NO: 20). Lanes 5 and 6 contain *Y. pestis* KIM8-3002 harboring a deletion in the *yscF* gene. After the 2 h growth, the culture was shifted to 37°C to induce expression of the Ysc type III secretion system and the Low Calcium Response. Following 4 h of growth at 37°C, cultures were centrifuged to obtain whole cell fractions and cell-free culture supernatant fractions.

[0051] Total proteins from each fraction were precipitated with 10% tri-chloro acetic acid. Dried proteins were re-suspended in SDS-PAGE sample buffer and electrophoresed in a 15% SDS-PAGE gel. Proteins were transferred to an Immobilon membrane (Millipore, Bedford, MA) and immuno-blotted with pooled mouse serum used at a 1:20,000 dilution. Mouse serum was obtained by bleeding mice subsequent to immunization with HT-YscF. Immunoblots were blocked in 5% non-fat skim milk in 1X Tris-buffered saline plus 0.05% Tween-20 (TTBS). Pooled serum was added to 1% non-fat dry skim milk in 1% TTBS and incubated overnight. Detection of bound antibody was accomplished by incubation with an alkaline phosphatase conjugated goat-anti-mouse antibody. Antibody complexes were visualized by adding NBTBCIP.

[0052] Serum from several mice were pooled to control for animal specific variation. The position and sizes for the molecular weight markers are indicated and the position of YscF is shown (FIG. 5). As seen in FIG. 5, YscF is visualized on the immunoblot as a highly reactive band of the correct size predicted for YscF and the band is only seen in strains containing the *yscF* gene. Importantly, no band is seen in lanes 5 and 6 that contain proteins derived from the *yscF* deletion strain. In lanes 1 and 2, calcium regulation of the YscF band is seen as expected. The higher molecular weight bands seen in the whole cell fraction represent cross-reactive *Y. pestis* bands that are present in samples probed with pre-immune serum (not shown). The higher molecular weight band seen the culture supernatant fractions is consistent with the expected size of an YscF trimer.

Example X.

[0053] In another exemplary embodiment, antibodies or derivatives thereof (*e.g.*, fragments such as Fab, $F(ab')_2$ or Fv fragments), which are directed against isolated or recombinant YscF or homologs thereof are used in passive immunotherapy, diagnostic immunoassays and in the

generation of anti-idiotypic antibodies. Serum including polyclonal antibodies of derivatives thereof directed against the isolated or recombinant YscF or homologs thereof is obtained as described herein. Monospecific antibodies directed against the isolated or recombinant YscF or homologs thereof are affinity purified from polyspecific antisera by a modification of the method of Hall et al. (*See*, Nature, 311, 379-387, 1984).

[0054] An epitope of the isolated or recombinant YscF or homologs thereof to which the antibodies bind is determined using known techniques including, but not limited to, Pepscan or microarray technology. When the amino acid residues of the epitope are determined, one skilled in the art generates peptides having amino acid residues of the epitope by artificially synthesizing the peptides of the epitope or using recombinant nucleic acid technology. The synthetic peptides are used to form a composition, vaccine or medicament and used to treat a disease associated with a pathogen of *Yersinia* origin or generating antibodies.

[0055] Monoclonal antibodies reactive against the isolated or recombinant YscF or homologs thereof are prepared by immunizing mice using techniques known in the art. (*See*, Kohler and Milstein, *Nature*, 256, 495-497, 1975). Hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin in an appropriate cell culture medium such as Dulbecco's modified Eagle's medium. Antibody producing hydridomas are cloned, such as by using the soft agar technique of MacPherson. (*See*, Soft Agar Techniques, Tissue Culture Methods and Applications, Kruse and Paterson, eds., Academic Press, 276, 1973). Discrete colonies are transferred into individual wells of culture plates for cultivation in an appropriate culture medium. Antibody producing cells are identified by screening with the appropriate immunogen. Immunogen positive hydriboma cells are maintained by techniques known in the art and specific anti-monoclonal antibodies are produced by cultivating the hydridomas *in vitro* or preparing ascites fluid in mice following hydridoma injection using procedures known in the art.

[0056] Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the isolated or recombinant YscF or homologs thereof of the pathogen against which protection is desired and are used as an immunogen in a vaccine as described in Dreesman et al. (*See, J. Infect. Disease*, 151, 741, 1985). Techniques for raising anti-idiotypic antibodies are known in the art. (*See*, MacNamara et al., *Science* 226, 1325, 1984).

Example XI.

[0057] A diagnostic kit including antibodies generated against isolated or recombinant YscF or homologs thereof for diagnosing disease is also included. The kit contains at least one antibody or fragment thereof directed against the isolated or recombinant YscF or homologs thereof. The immunochemical reaction employed using the kit is a sandwich reaction, an agglutination reaction, a competition reaction or an inhibition reaction, all of which are known by those of ordinary skill in the art. When the kit is used to perform a sandwich reaction, the kit includes isolated or recombinant YscF or homologs thereof bonded to a solid support, such as the inner wall of a tube or well of a plate. The kit is used to detect the presence of isolated or recombinant YscF of *Yersinia* origin or homologs thereof in fleas, mice, rats, prairie dogs, pigs, humans, cats dogs and tissues thereof to ascertain if populations of the animals have been infected with pathogens of *Yersinia* origin.

[0058] The exemplary embodiments described herein are not meant to limit the scope of the present invention. The present invention may be carried out using embodiments different from those specifically exemplified herein. Therefore, the scope of the present invention is not limited by the exemplary embodiments, but is defined by the appended claims.

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